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# Retinoic acid receptors are required for skeletal growth, matrix homeostasis and growth plate function in postnatal mouse

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## ABSTRACT

The retinoic acid receptors  $\alpha$ ,  $\beta$  and  $\gamma$  (RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$ ) are nuclear hormone receptors that regulate fundamental processes during embryogenesis, but their roles in skeletal development and growth remain unclear. To study skeletal-specific RAR function, we created conditional mouse mutants deficient in RAR expression in cartilage. We find that mice deficient in RAR $\alpha$  and RAR $\gamma$  (or RAR $\beta$  and RAR $\gamma$ ) exhibit severe growth retardation obvious by about 3 weeks postnatally. Their growth plates are defective and, importantly, display a major drop in aggrecan expression and content. Mice deficient in RAR $\alpha$  and RAR $\beta$ , however, are virtually normal, suggesting that RAR $\gamma$  is essential. In good correlation, we find that RAR $\gamma$  is the most strongly expressed RAR in mouse growth plate and its expression characterizes the proliferative and pre-hypertrophic zones where aggrecan is strongly expressed also. By being avascular, those zones lack endogenous retinoids as indicated by previous RARE reporter mice and our direct biochemical measurements and thus, RAR $\gamma$  is likely to exert ligand-less repressor function. Indeed, our data indicate that: aggrecan production is enhanced by RAR $\gamma$  over-expression in chondrocytes under retinoid-free culture conditions; production is further boosted by co-repressor Zac1 or pharmacologic agents that enhance RAR repressor function; and RAR/Zac1 function on aggrecan expression may involve Sox proteins. In sum, our data reveal that RARs, and RAR $\gamma$  in particular, exert previously unappreciated roles in growth plate function and skeletal growth and regulate aggrecan expression and content. Since aggrecan is critical for growth plate function, its deficiency in RAR-mutant mice is likely to have contributed directly to their growth retardation.

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## Introduction

Prenatal and postnatal growth of the skeleton is a complex and tightly-regulated spatio-temporal process. In most skeletal elements, growth is attributable to the activity of the growth plates where chondrocytes proliferate, enlarge into pre-hypertrophic and hypertrophic cells, and are ultimately replaced by endochondral bone and marrow (Wilsman et al., 1996). Growth plate function is directed and orchestrated by multiple systemic and local factors that include growth hormone, hedgehog proteins, and Runx and Sox transcription factors (Iwamoto et al., 1999; Lefebvre and Smits, 2005; Nilsson

et al., 2005). In addition, functioning of growth plates depends on their extracellular matrix composed of aggrecan, collagen II and other macromolecules (Schwartz and Domowicz, 2002; van der Eerden et al., 2003). Indeed, abnormalities in matrix gene expression and structure can cause growth plate malfunction and chondrodysplasias including dwarfism (ul Haque et al., 1998; Watanabe et al., 1994). For example, the lethal mouse chondrodystrophy Cartilage Matrix Deficiency (*cmd*) is caused by stop codon mutations in aggrecan gene; the mutant mice exhibit short limbs, an enlarged abdomen and cleft palate, and their growth plates are disorganized (Watanabe et al., 1994). In addition, aggrecan expression levels and turn-over rates are tightly regulated and important for normal growth plate function (Shapses et al., 1994; Wang et al., 2004). Clearly, a normal complement of matrix components is critical for growth plate function and skeletal growth, but it is far from clear particularly at the molecular level how matrix homeostasis is established and maintained.

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The retinoic acid receptors  $\alpha$ ,  $\beta$  and  $\gamma$  (RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$ ) are nuclear hormone receptors that regulate fundamental processes during development (Chambon, 1996; Sucov and Evans, 1995). The RARs form heterodimers with RXR $\alpha$ , RXR $\beta$  and RXR $\gamma$ , interact with specific DNA response elements (RAREs) and can act as ligand-dependent transcriptional activators or ligand-less transcriptional repressors. When ligands are present, a conformational change allows interaction with co-activators and proteins with histone acetyltransferase (HAT) activity (Glass and Rosenfeld, 2000; Moras and Gronemeyer, 1998). When ligands are absent, the RAR-RXR dimers bound to RAREs recruit co-repressors, including nuclear receptor corepressor-1 (NCoR-1) and silencing mediator for retinoic acid and thyroid hormone receptors (SMRT/NCoR-2) (Chen et al., 1996; Horlein et al., 2002), and additional factors such as mSin3A and histone deacetylases (HDACs), thus promoting chromatin compaction and transcriptional repression. Studies by Chambon and coworkers first established that RARs are differentially expressed during early steps of limb skeletogenesis and required for skeletal development (Mendelsohn et al., 1992). While single RAR-null mice were found to be viable and displayed few defects, double-null mice lacking RAR $\alpha$ /RAR $\gamma$  or RAR $\beta$ /RAR $\gamma$  died before or immediately after birth and displayed severe skeletal defects including homeotic transformations, growth retardation and malformation of limb bones (Lohnes et al., 1994). It has remained largely unclear ever since, however, what specific roles the RARs have in mammalian skeletal development and growth plate functioning. To address this important issue, we created conditional double mutant mice deficient in RAR $\alpha$ /RAR $\beta$ , RAR $\alpha$ /RAR $\gamma$  or RAR $\beta$ /RAR $\gamma$  in cartilage. We find that mice deficient in RAR $\alpha$ /RAR $\gamma$  or RAR $\beta$ /RAR $\gamma$  exhibit significant skeletal growth retardation and growth plate abnormalities, whereas mice deficient in RAR $\alpha$ /RAR $\beta$  are essentially normal. The apparent centrality of RAR $\gamma$  is reaffirmed by our extensive *in vitro* experiments with wild type and RAR-deficient chondrocytes in which we have also identified Zc1 as a likely RAR functional partner. The data provide novel insights into the molecular regulation of growth plate function and skeletal growth.

## Materials and methods

### RAR-deficient mice and genotyping

Floxed RAR mice (RAR $\alpha^{f/f}$ , RAR $\beta^{f/f}$  or RAR $\gamma^{f/f}$ ) (Chapellier et al., 2002a,b,c) were mated with Col2a1-Cre mice (Ovchinnikov et al., 2000) to eventually create homozygous double RAR-deficient mice in cartilage. Briefly, Col2a1-Cre mice were first mated with a single RAR floxed homozygous mouse to create heterozygous Col2a1-Cre<sup>+</sup>/RAR<sup>f/+</sup> mice that were in turn mated to produce Col2a1-Cre<sup>+</sup>/RAR<sup>f/f</sup> mice deficient in one RAR in cartilage. To generate double mutant mice, single RAR deficient mice were mated to each other to generate double heterozygous mice (Col2a1-Cre<sup>+</sup>/RAR<sup>f/+</sup>/RAR<sup>f/+</sup>). Resulting mice, usually Col2a1-Cre<sup>+</sup>/RAR<sup>f/f</sup>/RAR<sup>f/+</sup>, were mated to obtain double RAR-deficient mice (Col2a1-Cre<sup>+</sup>/RAR<sup>f/f</sup>/RAR<sup>f/f</sup>) in all receptor combinations ( $\alpha/\beta$ ,  $\alpha/\gamma$ ,  $\beta/\gamma$ ). Cre-mediated excision deletes exon 8 in RAR $\alpha$  and RAR $\gamma$  that encodes most of DNA binding domain, and deletes exons 9 and 10 in RAR $\beta$  that encode much of the ligand-binding domain. In each case, deletions also cause a frameshift mutation, thus resulting in functional ablation of all splice forms. Genotyping procedures and results are shown as Supplementary Figs. S1 and S2.

### In situ hybridization, anatomical measurements and RT-PCR

In situ hybridization was carried out with radiolabeled riboprobes (Iwamoto et al., 2000; Koyama et al., 1999). Plasmids were: pGEM-T encoding 548–1627 of NM\_009024 for RAR $\alpha$ ; pGEM-T encoding 1480–2116 of XM\_127583 for RAR $\beta$ ; pGEM-T encoding 1673–2628 of NM\_011244 for RAR $\gamma$  that recognize all RAR $\gamma$  splice forms. Col9a1,

Sox 9, aggrecan and H4C probes were described previously (Tamamura et al., 2005). Dark and bright field images were captured using a digital camera. In some experiments, dark field images were pseudo-colored using Adobe Photoshop software.

Mice sedated by brief isoflurane exposure were measured from nose to tail base to nearest 1/10 cm. Femurs were dissected out and distance from greater trochanter to the medial condyle was measured with digital calipers to nearest 1/100 mm.

Tibias and vertebrae at L3 level collected from 3- and 5-week-old double RAR $\beta$ /RAR $\gamma$ -deficient and double floxed littermates were fixed in 4% paraformaldehyde, decalcified in 10% EDTA (pH 7.4) for three weeks at 4 °C, and embedded in paraffin. Control and mutant sections mounted on same slides were stained with 0.01–0.1% Safranin-O/2% fast green or 0.1% Alcian blue pH 1.0. Staining was as brief as possible to avoid dye saturation. Real-time PCR was used to compare levels of transcriptional co-factors using RNA isolated from newborn tibial and femoral epiphyseal cartilage. Primer pairs are listed in Table S2 and genes tested were the following: NCoR1 (nuclear receptor co-repressor 1) (Jepsen et al., 2000); NCoR2 (also called SMRT) (Hauksdottir et al., 2002); Lcor (ligand-dependent nuclear receptor corepressor) (Fernandes et al., 2003); PRAME (preferentially expressed antigen in melanoma) (Epping et al., 2005); NSD1 (nuclear receptor binding SET domain protein 1) (Huang et al., 1998); Sorbs3 (sorbin and SH3 domain containing protein 3) (Bour et al., 2005); Mx1 (msh homeobox 1) (Weston et al., 2003); and Mta1 (metastasis-associated 1) (Mazumdar et al., 2001).

To analyze chondrocyte proliferation, serial sections were prepared from the proximal tibial growth plates of 3- and 5-week-old double RAR $\beta$ /RAR $\gamma$ -deficient and double floxed littermates. Sections were hybridized with radiolabeled H4C riboprobes and then stained with eosin. Bright and dark field pictures were taken from a minimum of 6 slides from each genotype and age then printed. Each picture was used to manually count every cell present in the proliferative zone, defined as the tissue area above the *Indian hedgehog*-expressing prehypertrophic zone. The same process was repeated by determining the number of H4C-positive cells; positive signal was defined as that given by a minimum 5 hybridization grains per cell. Statistical significance was determined by two-tailed unpaired Student's *t*-test.

### Microcomputed tomography ( $\mu$ CT) analysis

Tibia and femurs were analyzed by  $\mu$ CT at 45 kVp. Trabecular bone volume, connectivity, trabecular thickness and mineral density were analyzed and quantified with a Scanco  $\mu$ CT 40 apparatus using proprietary software.

### Chondrocyte cultures

Cartilage fragments from neonatal epiphyses and 3 to 5-week old ribs were washed with Ca/Mg-free HBSS plus antibiotics and with 0.05% trypsin/0.01% EDTA. Fragments were incubated in fresh trypsin/EDTA mixture at 37 °C for 20 min, washed and incubated with 0.1% testicular collagenase in  $\alpha$ MEM for 3–4 h. Neonatal mouse cartilage tissue was dispersed by pipetting, and single cells were inoculated onto type I collagen-coated multiwell dishes maintained in 10% FBS in  $\alpha$ MEM. Rib cartilage fragments from 3 and 5 weeks old mice were partially digested with the initial collagenase treatment. We found that more than 4 h collagenase treatment not only damaged cell viability, but also decreased yield of chondrocytes. To avoid those problems, we maintained partially digested cartilage fragments for 7–10 days in 10% FBS and 1 ng/ml recombinant basic FGF (R&D) and then digested again with 0.1% collagenase for 2–3 h. Ascorbic acid (10  $\mu$ g/ml) was added to the medium after cells reached confluency. Cartilage matrix content was determined by Alcian blue staining (pH 1.0).



## Transfections

### Single reporter assay

$4 \times 10^4$  cells per well were seeded onto a type I collagen coated 96 wells plate the day before transfection. Cells were maintained in 100  $\mu$ l 10% FBS in MEM. Medium was changed and cells were immediately transfected with 0.2  $\mu$ g/well of retinoic acid response element (RARE) luciferase reporter vector (Panomics, CA) and 0.2  $\mu$ l of Lipofectamine LTX (Invitrogen, CA) according to the manufacturer's protocol. Cultures were treated with indicated doses of all-trans retinoic acid (RA) (Sigma), RAR $\gamma$  agonist (VTP 204647) or RAR inverse agonist (INV) (AGN/VTP 194310) for 24 h after transfection; parallel control cultures received vehicle alone. Forty-eight hours later, cells were subjected to luciferase assay.

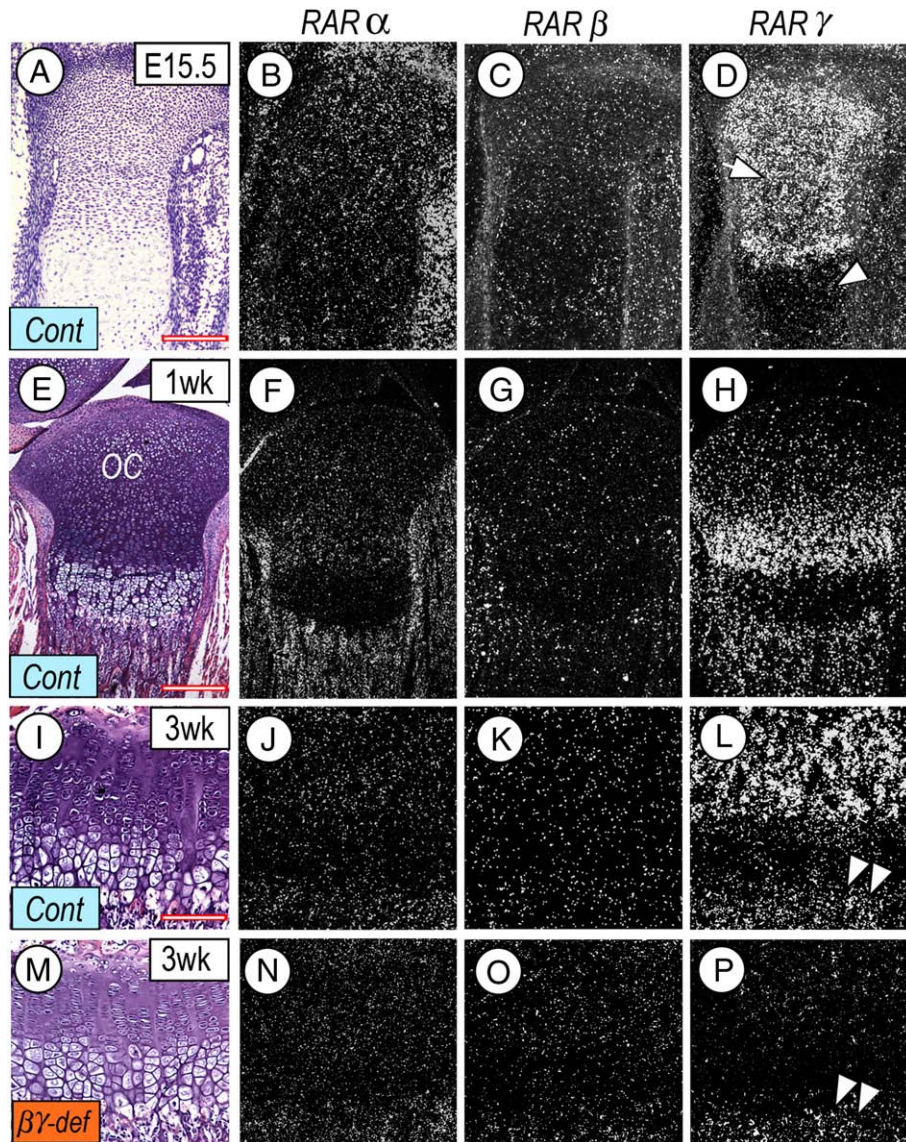
### Dual reporter assay

A dual reporter assay system was employed to study the function of individual RARs and Zac1. Cell seeding and maintenance conditions were the same as those of the single reporter assay. For gain of function experiments, 0.1  $\mu$ g each of expression vector and RARE-luc

and 1 ng phRG-SV40 (Promega) were co-transfected with 0.2  $\mu$ l of Lipofectamine LTX. For loss of function experiments, 0.2 pmol of siRNA (non-specific siRNA mixture D-001810-10; SMARTpool siRNA mixture targeting mouse Zac1 (Plagl1), E-046206; RAR $\alpha$ , E-047625 and RAR $\gamma$ , E-040974, Dharmacon, CO), 30 ng RARE-luc and 1 ng phRG-SV40 (Promega) were transfected to each well with 0.2  $\mu$ l Lipofectamine 2000 reagent (cat. No. 25087w pps, Invitrogen). Some cultures were treated with various retinoid derivatives from 24 to 48 h after transfection. Forty-eight hours after transfection, cells were harvested and subjected to dual luciferase assay (Dual Luciferase Reporter Assay System, E1910, Promega). Transfection efficiency was normalized by *Renilla* luciferase activity generated by phRG-SV40.

### Protein functional interaction assays

Interactions between RAR $\gamma$  and Zac1 were tested with Promega Checkmate mammalian two-hybrid system. RAR $\gamma$  and Zac1 subcloned to pACT and pBIND vectors respectively were transfected to AD293 cells with Lipofectamine LTX reagent. Total 0.2  $\mu$ g of pACT, pBIND and pG5-luc (GAL4-luc reporter vector) were transfected at a ratio of 4:4:2.



**Fig. 1.** RAR expression patterns in long bone growth plates. (A–L) In situ hybridization analysis of RAR $\alpha$ , RAR $\beta$  or RAR $\gamma$  expression using sections of wild type E15.5 (A–D), 1 week-old (E–H) and 3 week-old (I–L) proximal tibial growth plates and 3 week-old RAR $\beta$ /RAR $\gamma$ -deficient tibial growth plates (M–P). In (D), arrow points to upper growth plate zones, while arrowhead points to hypertrophic zone. In (L, P), double arrowheads point to primary spongiosa. OC, secondary ossification center. Size bars: A–D, 200  $\mu$ m; E–H, 300  $\mu$ m; and I–P, 75  $\mu$ m.

### Proteoglycan analysis

Newborn epiphyseal chondrocytes seeded at  $2 \times 10^4$  cells/6 mm well were maintained in 10%FBS/ $\alpha$ MEM. When confluent, cultures were pre-incubated for 24 h in  $\alpha$ MEM/0.3%FBS, treated with various retinoid concentrations for 4 h and labeled with 5  $\mu$ Ci/ml  $^{35}$ S-sulfate for 20 h. Proteoglycan synthesis was determined by measuring incorporation of  $^{35}$ S-sulfate into materials precipitated with cetylpyridinium chloride after protease digestion (Takebayashi et al., 1995).

To test RAR $\gamma$  or Zc1 over-expression on proteoglycan synthesis, chondrocytes were transfected with RAR $\gamma$  (pCMV-HA-RAR $\gamma$ ), Zc1 (pCMV-Zc1) or insert-less expression vector using Lipofectamine LTX reverse transfection protocol in which 0.1  $\mu$ g of DNA and 0.4  $\mu$ l Lipofectamine LTX in 20  $\mu$ l Opti-MEM medium were dispensed into each well. Freshly isolated chondrocytes ( $5 \times 10^4$  cells) suspended in 10% FBS/ $\alpha$ MEM medium were seeded onto the pre-coated wells. Six hours later, medium was changed to retinoid-depleted medium (0.5% charcoal-treated serum, 1 $\times$  ITS mixture and 0.5% BSA in DMEM) and cultures were treated with retinoids or left untreated. Cells were lysed 24–48 h after transfection for the reporter gene assay or labeled with 5  $\mu$ Ci/ml of  $^{35}$ S-sulfate for 20 h to measure proteoglycan synthesis.

The parallel cultures that received identical treatment were used to determine DNA contents (Johnson-Wint and Wellis, 1982).

### Analysis of endogenous retinoids

This procedure was carried out as described (Kane et al., 2005, 2008). Working in a dark room to protect endogenous retinoids from light, specimens of upper growth plate zones were isolated from rabbit rib chondral-osseous junction by microsurgery using a dissection microscope equipped with yellow filters. Absolute care was taken to free the fragments of adjacent perichondrium and other tissues. Liver fragments were collected from same animals to serve as internal positive controls. After measuring wet weight of each sample, tissue fragments were homogenized by several 5–10 s pulses with a Polytron homogenizer on 1/4 maximum power, and samples were analyzed and quantified by LC-MS/MS using an API-4000 (Applied Biosystems) with atmospheric pressure chemical ionization (APCI) in positive ion mode.

## Results

### RAR gene expression patterns

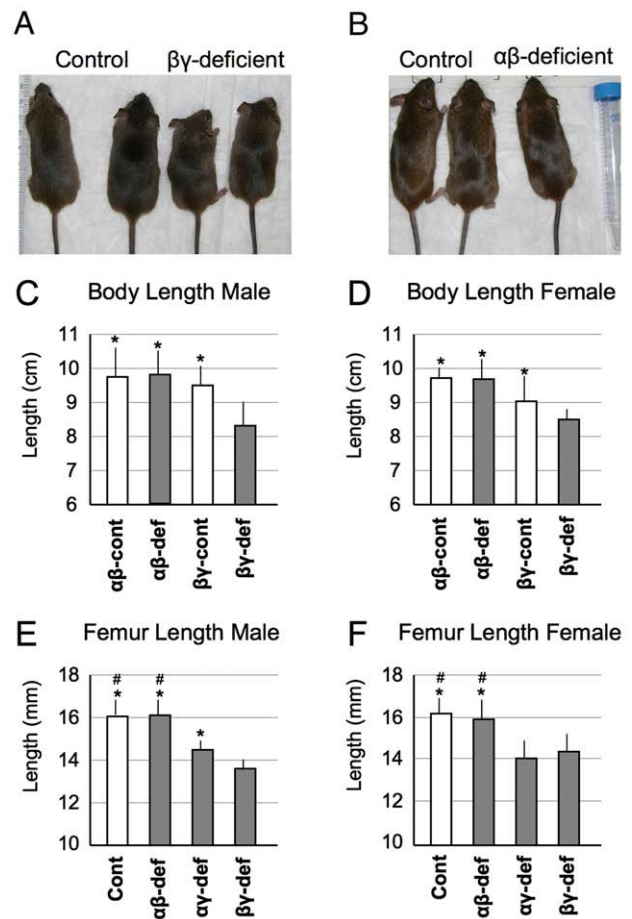
At the outset of this project, we determined the gene expression patterns of RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$  in mouse limb growth plates, information that, surprisingly, was still lacking in the literature. In E15.5 embryos, RAR $\alpha$  and RAR $\beta$  were mildly expressed (Figs. 1A–C), but RAR $\gamma$  was very strongly expressed in resting, proliferative and pre-hypertrophic zones (Fig. 1D, arrow) and was down-regulated in hypertrophic zone (Fig. 1D, arrowhead). Similar overall patterns were seen in tibial growth plates in 1 week- and 3 week-old mice (Figs. 1E–H and I–L, respectively), and there was also appreciable RAR $\alpha$  expression in perichondrium (Fig. 1F) and RAR $\gamma$  expression in primary spongiosa (Fig. 1L, arrowheads). Clearly, the RARs are differentially expressed in mammalian growth plates and RAR $\gamma$  is the most abundantly expressed family member.

### Conditional compound RAR-deficient mice

To determine RAR roles in growth plate, we produced double RAR-deficient mice in cartilage by mating floxed RAR mice with *Col2a1-Cre* mice (Ovchinnikov et al., 2000) (all with mixed 129/SvPas-C57Bl/6 background). Mutant mice were born at Mendelian ratios. To determine efficiency and specificity of RAR deletion, DNA samples from rib and limb growth plate cartilaginous fragments and liver from

newborn wild type (WT) and double RAR-deficient mice were processed for genomic PCR, using primers to distinguish wild type (W), floxed (F) and null (N) alleles (Fig. S1). RAR genes had been deleted in mutant cartilage, but not liver (Fig. S1). Efficiency of RAR $\gamma$  ablation in double RAR $\alpha$ /RAR $\gamma$ - or RAR $\beta$ /RAR $\gamma$ -deficient cartilage was nearly complete (Fig. S1), whereas efficiency of RAR $\alpha$  and RAR $\beta$  ablation averaged over 80% (Fig. S1). Southern hybridization also showed that RAR genes were deleted in cartilage, but not heart, liver, brain, spleen and bone marrow DNA; deletion efficiency was over 95% for RAR $\gamma$  and about 80% for RAR $\alpha$  and RAR $\beta$  (Fig. S2). In situ hybridization on tibial sections from 3 week-old RAR $\beta$ /RAR $\gamma$ -deficient mice (Figs. 1M–P) confirmed that RAR $\gamma$  gene expression was markedly reduced in growth plate (Fig. 1P), but signal was still appreciable over primary spongiosa (Fig. 1P, arrowhead).

Given that mutant mice were born at Mendelian frequency and appeared normal at birth, we closely monitored them throughout maturation. Starting at about 3 weeks, we noted that RAR $\alpha$ /RAR $\gamma$ - and RAR $\beta$ /RAR $\gamma$ -deficient mice were consistently smaller than control littermates (heretofore, control littermates represent double floxed RAR mice lacking *Col2a1-Cre*) (Fig. 2A). By 2 months, overall growth retardation was pronounced in both mutant females and males and



**Fig. 2.** Physical appearance and skeletal growth in control and RAR-deficient mice. (A) The two RAR $\beta$ /RAR $\gamma$ -deficient 5 week-old mice on the right (indicated as  $\beta\gamma$ -deficient) are visibly smaller than their control littermates on the left. (B) No obvious difference in body size is appreciable in 5 week-old control versus RAR $\alpha$ /RAR $\beta$ -deficient littermates. (C–F) Average body and femur lengths in male and female control and dual RAR-deficient mice at 8 weeks of age. Body length was measured from nose tip to sacral area. Numbers of mutant mice analyzed in this experiment are: RAR $\alpha$ /RAR $\beta$ -deficient ( $\alpha\beta$ -def,  $n = 13$ ) and control littermate ( $\alpha\beta$ -cont,  $n = 19$ ), RAR $\beta$ /RAR $\gamma$ -deficient ( $\beta\gamma$ -def,  $n = 24$ ) and its control ( $\beta\gamma$ -cont,  $n = 32$ ), and RAR $\alpha$ /RAR $\gamma$ -deficient ( $\alpha\gamma$ -def,  $n = 13$ ) and its control littermate ( $n = 14$ ). Control in E and F are total of all three control littermates ( $n = 65$ ). Obtained data were subjected to the Mann–Whitney test. \* $p < 0.05$  compared to  $\beta\gamma$ -def; # $p < 0.05$  compared to  $\alpha\gamma$ -def.



amounted to about 25% over controls (Figs. 2C, D). This trend was confirmed in limb skeletal elements such as femurs (Figs. 2E, F). Notably, mice deficient in *RARα/RARβ* genes were virtually similar to control littermates in both body size (Fig. 2B) and skeletal element length (Figs. 2C–F). In total, we examined over 115 mice from various litters (see Fig. 2 legend for details) and the above phenotypic changes in *RARα/RARγ*- and *RARβ/RARγ*-deficient mice were invariably seen. Clearly, deficiency of *RARα/RARγ* or *RARβ/RARγ* genes in cartilage causes significant skeletal growth retardation, whereas *RARα/RARβ* deficiency is tolerated.

#### Growth plate organization and function

What could account for the growth retardation? Normally, growth plates sustain skeletal growth by: (a) proliferation of chondrocytes; (b) matrix synthesis and accumulation; and (c) chondrocyte hypertrophy (Hunziker, 1994; Wilsman et al., 1996). To determine whether and which of these parameters was affected, longitudinal serial sections of femoral and tibial growth plates from 3- and 5-week-old control and *RARβ/RARγ*-deficient littermates were processed for histological, histochemical and gene expression analyses. Interestingly, the height of mutant growth plates (Figs. 3B, D, vertical green line) was consistently shorter than that of control littermates at both ages (Figs. 3A, C, vertical green line). Shortening appeared to affect primarily the upper zones (Figs. 3B, D, vertical red line), while the hypertrophic zone seemed largely unaffected.

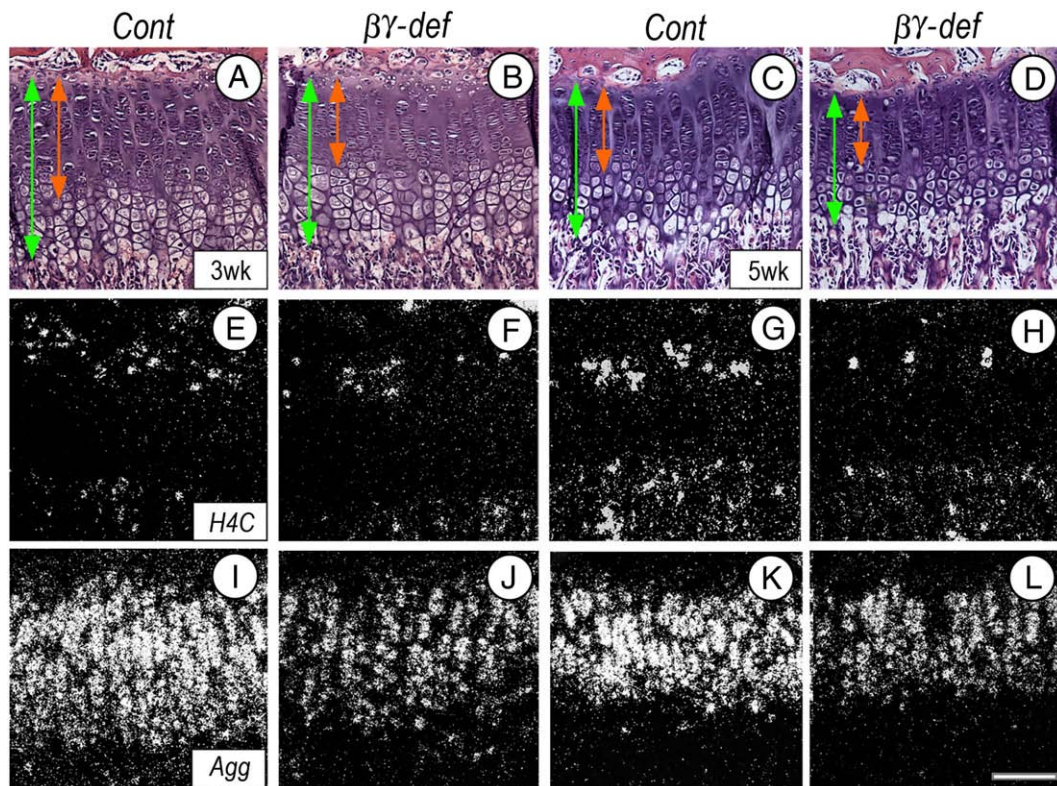
To assess proliferation, companion sections were processed for close histological assessment and for gene expression of histone 4C (*H4C*), a general marker of mitotic cells. The average number of chondrocytes present in the proliferative zone in control 3- and 5-week-old tibial growth plates was  $180 \pm 13$  and  $179 \pm 5$ , respectively. Interestingly, this number had decreased to  $151 \pm 15$  and  $150 \pm 11$  in

*RARβ/RARγ*-deficient 3- and 5-week-old tibial growth plates ( $p < 0.01$ ). A similar trend was observed in companion sections processed for *H4C* expression; fewer positive chondrocytes were present in mutant proliferative zone (Figs. 3F, H, arrowheads) than control (Figs. 3E, G, arrows).

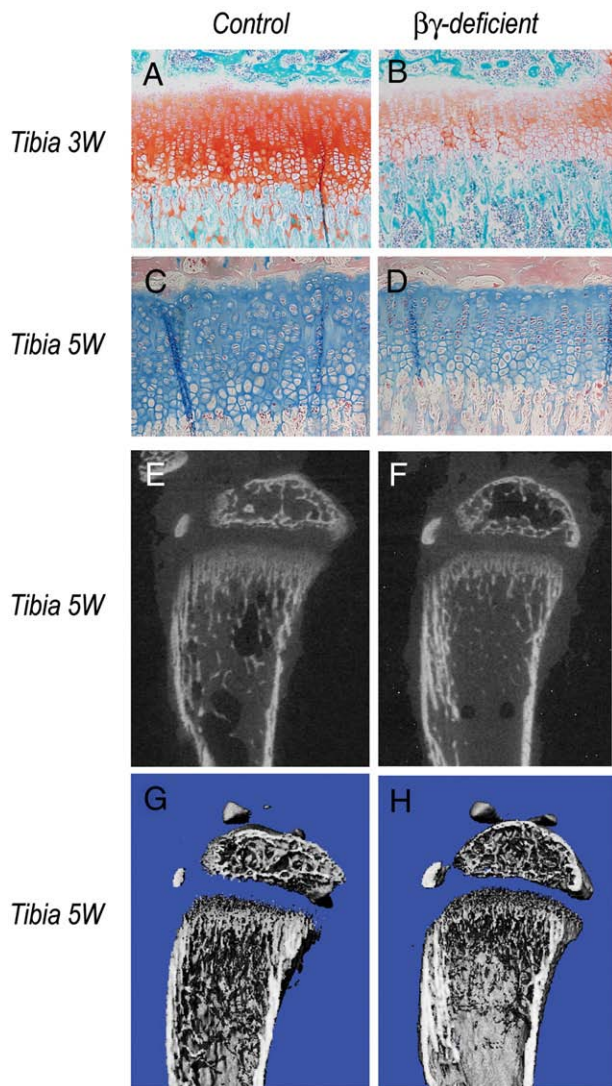
To monitor matrix synthesis and accumulation, we focused on aggrecan given its essential role in growth plate function. Aggrecan transcripts were extremely abundant in control growth plates, particularly in proliferative and pre-hypertrophic zones (Figs. 3I, K). In contrast, aggrecan expression was clearly reduced in *RARβ/RARγ*-deficient growth plates (Figs. 3J, L). These hybridizations were conducted with control and mutant growth plate sections mounted and processed on the same glass slides; thus, hybridization signal is directly comparable.

To visualize proteoglycans, growth plate sections from 3-week-old control and mutant littermates mounted on the same glass slides were stained with Safranin-O or Alcian blue. In controls, staining was strong (Figs. 4A, C), but was clearly lower in *RARβ/RARγ*-deficient growth plates (Figs. 4B, D). Decreases in proteoglycan content were also observed at other sites in the same mutant animals including vertebral endplates and annulus fibrosus (Fig. S3).

Given that growth plate function is also required for endochondral ossification (Chan and Jacenko, 1998; Jacenko et al., 1993), we predicted that the above growth plate defects, and the decrease in aggrecan content in particular (Poole et al., 1982), should impair trabecular bone formation. Thus, we subjected tibias and femurs from control and *RAR*-deficient littermates to micro-computed tomography ( $\mu$ CT) to reveal mineralized tissue. Indeed, there was a clear trabecular bone reduction in *RARβ/RARγ*-deficient mice (Figs. 4E–H) and *RARα/RARγ*-deficient mice (Table S1), while bone structure and quality were essentially normal in *RARα/RARβ*-deficient mice (Table S1).



**Fig. 3.** Analyses of growth plate organization, chondrocyte proliferation and aggrecan expression. (A–L) Serial sections from proximal tibial growth plates of 3 week-old (A, B, E, F and I, J) or 5 week-old (C, D, G, H and K, L) control and *RARβ/RARγ*-deficient littermates were processed for histological analysis (A–D) and in situ hybridization for proliferation marker *H4C* (E–H) or aggrecan (Agg) (I–L). Note the reduction of mutant growth plate height (vertical green line) mostly affecting upper zones (red line), and more obvious in 5 week-old mice. Size bar, 75  $\mu$ m.



**Fig. 4.** Histochemical and  $\mu$ CT analyses of long bones. (A–D) Longitudinal sections of proximal tibial long bone growth plates from 3 and 5 week-old control and  $RAR\beta/RAR\gamma$ -deficient littermates were processed for histochemical proteoglycan staining with safranin O or Alcian blue. Note the significant reduction in staining in mutant growth plates. (E–H) Long bones from 5 week-old control and  $RAR\beta/RAR\gamma$ -deficient littermates were scanned by  $\mu$ CT and viewed as 2D pictures (E, F) or 3D cut-plane pictures (G, H). Note the significant decrease in trabecular bone in mutant samples.

In summary, RARs are needed to sustain normal growth plate and chondrocyte function and bone formation and in particular, promote aggrecan expression and content.

#### RARs and proteoglycan expression

If RARs do benefit aggrecan gene expression and production, over-expression of RARs, and  $RAR\gamma$  in particular, should increase such parameters. It should be noted that endogenous active retinoids are largely absent in growth plate upper zones (where  $RAR\gamma$  and aggrecan are strongly co-expressed) as indicated by analysis of RARE reporter mice (von Schroeder and Heersche, 1998) and reiterated by our direct LC/MS/MS measurements (Fig. S4). Thus,  $RAR\gamma$  should influence aggrecan expression as ligand-less factor. To test these interesting possibilities, epiphyseal chondrocytes from newborn wild type mice were transfected with a CMV-driven expression plasmid encoding full-length mouse  $RAR\gamma$  (HA tagged) or control empty vector. Cells were maintained in medium containing 0.5% charcoal-stripped retinoid-free serum (Koyama et al., 1999) and were pulse-labeled

with  $^{35}$ S-sulfate to measure proteoglycan production. Clearly, proteoglycan synthesis increased over 30% in  $RAR\gamma$  transgene-expressing cultures compared to controls (Fig. 5A). Chondrocytes isolated from 3- to 4-week old ribs produced identical data (not shown). To confirm above data, chondrocytes from  $RAR\beta/RAR\gamma$ -deficient mice were subjected to same experimentation. Over-expression of  $RAR\gamma$  transgene did increase proteoglycan synthesis in these mutant cultures as well (Fig. 5A). Interestingly, baseline proteoglycan synthesis in untreated  $RAR\beta/RAR\gamma$ -deficient cultures was consistently lower than wild-type cultures, likely reflecting lower endogenous RAR gene levels in mutant cells (Fig. 5A).

When RARs are ligand-less, they function as transcriptional repressors (Koide et al., 2001; Kurosawa et al., 1995; Mannervik et al., 1999). Based on the data above, one would predict that experimental increase of RAR repressor function should lead to further increases in proteoglycan synthesis and levels. To test this intriguing idea, we resorted to the use of synthetic retinoids that are “inverse agonists”. Unlike standard agonists (that stimulate RAR transcriptional activity), inverse agonists stimulate RAR repressor function by reinforcing co-repressor binding (Germain et al., 2002; Gronemeyer et al., 2004; Klein et al., 1996; Thacher et al., 1999). Semi-confluent cultures in charcoal-treated medium were treated with 100 nM inverse agonist (INV) for 2 days; companion cultures were treated with vehicle (EtOH). Incorporation of  $^{35}$ S-sulfate did nearly double in treated versus untreated cultures (Fig. 5B, white histograms). Companion cultures were treated with 100 or 300 nM of inverse agonist for 6 days (fresh drug added every two days) to see whether this prolonged treatment would result in higher proteoglycan content. Quite clearly, prolonged treatment markedly increased the amount of Alcian blue-positive proteoglycan matrix compared to controls (Fig. 5I). This increase was appreciable also by the phase microscopy appearance of treated chondrocytes that were surrounded by a bright and highly refractive contour (generated by pericellular proteoglycan accumulation) (Fig. 5E) compared to the more muted appearance of untreated chondrocytes (Fig. 5C).

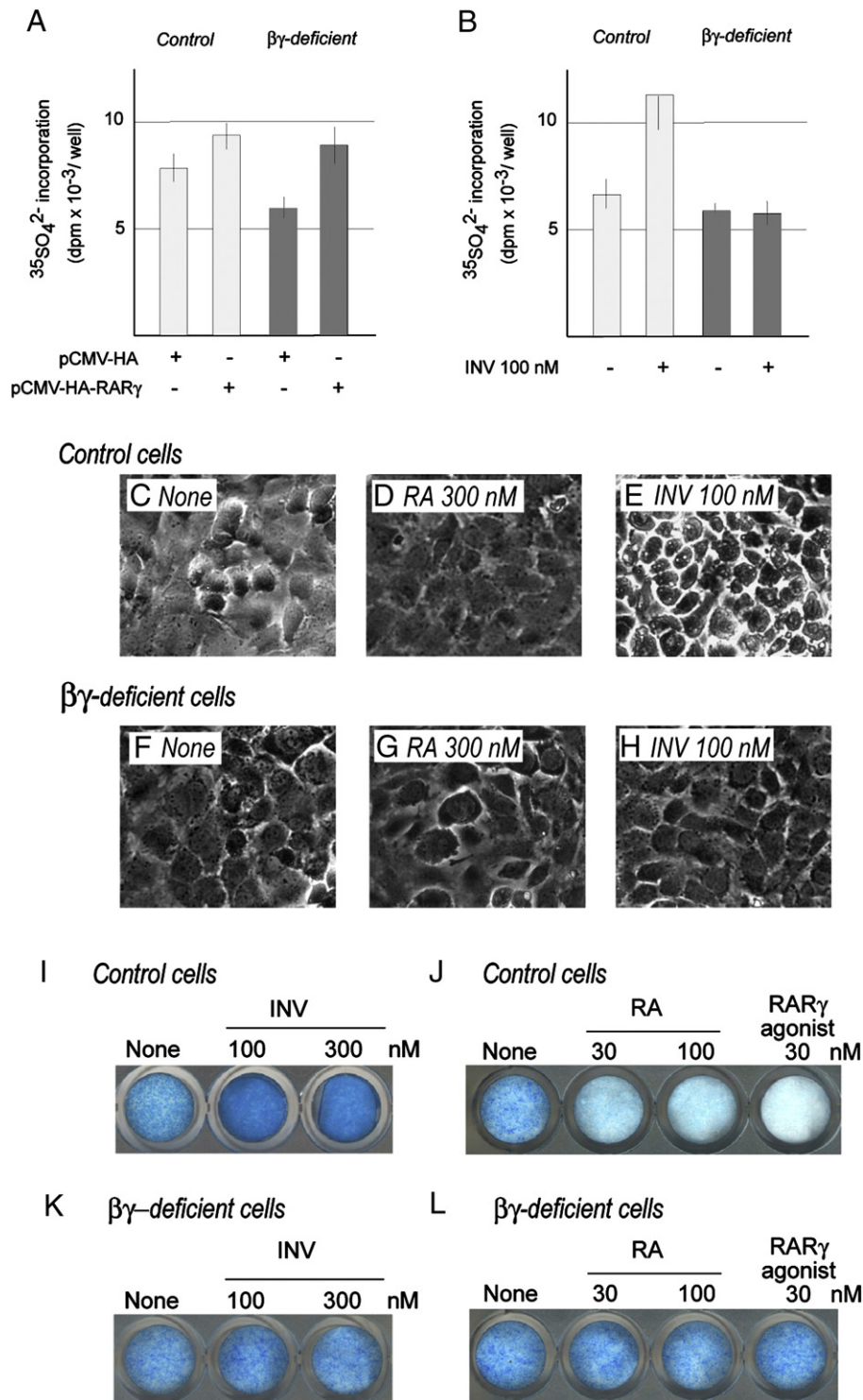
If these increases in proteoglycan synthesis and accumulation reflect inverse agonist-dependent stimulation of repressor/co-repressor function, one would predict that (a) the opposite should be seen in cultures treated with standard retinoid agonists that induce ligand-dependent RAR transcriptional activity; and (b) no effects should be seen in  $RAR$ -deficient cultures. In line with these predictions, treatment of control chondrocyte cultures with all-*trans*-retinoic acid (RA) or a  $RAR\gamma$  selective agonist: (i) drastically reduced proteoglycan accumulation (Fig. 5J); (ii) rendered the cells quite dull in appearance (Fig. 5D); and (iii) markedly decreased proteoglycan synthesis (not shown). In contrast, no appreciable changes in these parameters were seen in  $RAR\beta/RAR\gamma$ -deficient cultures that failed to respond to RA or  $RAR\gamma$  selective agonist (Figs. 5G, L) and inverse agonist (Figs. 5B, K).

Taken together, the above data strengthen the idea that RARs, and  $RAR\gamma$  in particular, are beneficial for proteoglycan expression in chondrocytes when operating in a retinoid-free condition and thus possibly as ligand-less repressors.

#### Searching for RAR co-repressors

Which factors could possibly serve as RAR co-repressors in cartilage? In other systems the most widely studied co-repressors are NCoR-1 or NCoR-2/SMRT (Hermanson et al., 2002; Jepsen et al., 2007), but their possible function in growth plate and skeletal growth is largely unknown. In addition, studies showed that ligand-less  $RAR\alpha$  does recruit NCoR-1 and NCoR-2 efficiently, but  $RAR\beta$  and  $RAR\gamma$  do not (Wong and Privalsky, 1998). Given that  $RAR\gamma$  is the most abundantly expressed RAR in growth plate, we searched databases to identify potential RAR co-repressor candidates using the following criteria: (1) candidate should associate with nuclear hormone

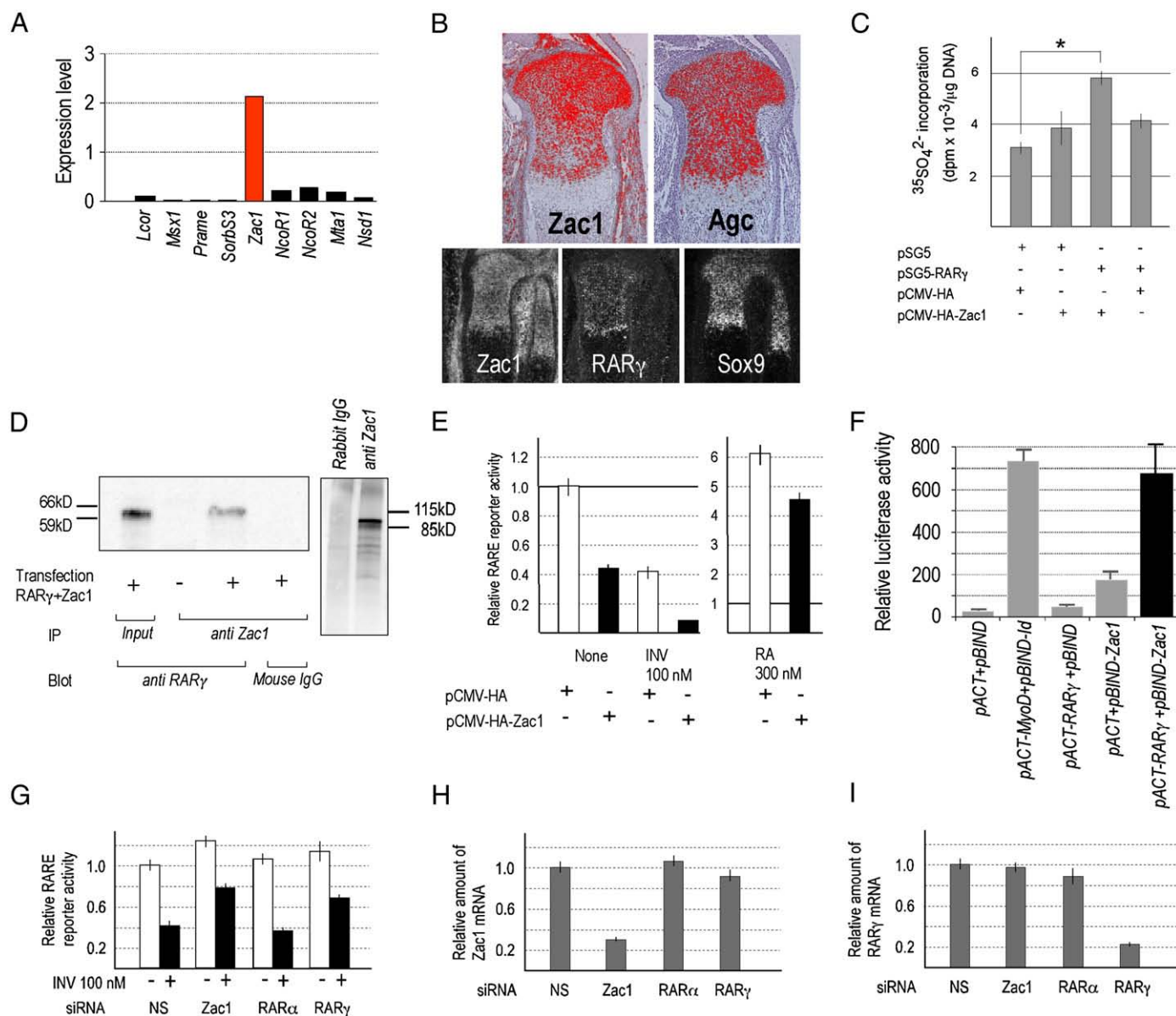




**Fig. 5.** Proteoglycan synthesis and content following *RAR $\gamma$*  over-expression and inverse agonist treatment. (A, B) Proteoglycan synthesis in control and *RAR $\beta$ /*RAR $\gamma$* -deficient chondrocyte cultures over-expressing *RAR $\gamma$*  (pCMV-HA-*RAR $\gamma$* ) or following treatment with inverse agonist. (C–H) Phase microscopic appearance of control and *RAR $\beta$ /*RAR $\gamma$* -deficient chondrocytes in untreated control cultures (None) and in companion cultures treated with 300 nM retinoic acid (RA) or 100 nM inverse agonist (INV). (I–L) Alcian blue staining of control and *RAR $\beta$ /*RAR $\gamma$* -deficient cultures treated for 6 days with inverse agonist, RA or a *RAR $\gamma$* -selective agonist. Consistency of results was established in three independent experiments.***

receptors and have repressor function; and (2) candidate should be expressed in skeletal tissue. This *in silico* search led us to *Zac1* (zinc finger protein which regulates apoptosis and cell cycle arrest) (Spengler et al., 1997). *Zac1* can act as transcriptional co-repressor or co-activator of nuclear hormone receptors (Huang and Stallcup, 2000) and interestingly, mice lacking *Zac1* exhibit growth retardation and altered bone formation (Varrault et al., 2006).

We found that *Zac1* is very strongly expressed in mouse growth plate cartilage; NCoR1 and NCoR-2 are expressed also, but at more than 5-fold lower levels (Fig. 6A). In situ hybridization on mouse growth plates confirmed that *Zac1* is strongly expressed and, strikingly, its expression pattern overlaps with that of *RAR $\gamma$* , *Sox9* and aggrecan (Fig. 6B). Indeed, proteoglycan synthesis in retinoid-free wild type chondrocyte cultures was stimulated by *RAR $\gamma$*  over-



**Fig. 6.** Analyses of *Zac1* expression patterns, interactions and function. (A) Real-time PCR analysis of transcriptional co-factors in newborn tibial and femoral epiphyseal cartilage. Expression levels are shown relative to GAPDH. (B) Expression patterns of *Zac1*, aggrecan (*Agc*), *RARγ* and *Sox9* in longitudinal sections of E15.5 mouse embryo tibias viewed at high (upper panels) and lower magnification (lower panels). (C) Proteoglycan synthesis in primary chondrocyte cultures following over-expression of *Zac1* and/or *RARγ*. Cultures maintained in retinoid-free medium were pulse-labeled with  $^{35}\text{S}$ -sulfate from 24 to 36 h after transfection. Graphs show average incorporation in triplicate cultures  $\pm$  SD. \* $p < 0.05$  by Student's *t*-test comparing control empty vector transfected cultures vs *Zac1* and *RARγ* transfected cultures. (D) Immunoprecipitation and immunoblot analysis of physical interaction between *RARγ* and *Zac1* (left panel). Specificity of *Zac1* antibodies was confirmed by immunoprecipitation of whole cell lysates compared to preimmune rabbit IgGs (right panel). (E) RARE reporter activity in primary cultured chondrocytes following *Zac1* over-expression with or without treatment with inverse agonist AGN (left panel). Similar analyses with or without treatment with retinoic acid (RA) (right panel). (F) Mammalian two hybrid assays showing that *RARγ* and *Zac1* interact to elicit very strong reporter activity (solid black column). *pACT*-*MyoD* and *pBIND*-*Id* were used as positive controls, and insert-less *pACT* and *pBIND* were negative controls. (G) RARE reporter activity in primary cultured chondrocytes following transfection of smart pool siRNA specific mixtures for *Zac1*, *RARα* or *RARγ* without (–) or with (+) co-treatment with 100 nM inverse agonist; non-specific (NS) was used in controls. (H, I) RT-PCR analysis of endogenous RNA levels for indicated genes following transfection of siRNAs for *Zac1* (H) or *RARγ* (I); note that only the targeted RNA was decreased in each case. Transfection efficiency of all reporter assays was normalized by *Renilla* luciferase activity generated by co-transfected *phRG*-SV40 vector. Two independent siRNA transfection experiments and three independent experiments with all the other transfections were carried out and produced similar results.

expression and was nearly doubled by over-expression of both *RARγ* and *Zac1* (Fig. 6C). To see that *Zac1* actually interacts with *RARγ*, we over-expressed *RARγ* and *Zac1* in AD293 cell line and processed whole cell extracts for *Zac1* immunoprecipitation followed by western blot for *RARγ*; clearly, *Zac1* associates with *RARγ* (Fig. 6D). To test whether *Zac1* influences RAR function at the RARE level, we co-transfected wild type chondrocyte cultures with an RARE reporter construct and a full-length mouse *Zac1* expression vector; parallel cultures received only the RARE reporter construct. Clearly, basal RARE reporter activity was decreased by *Zac1* over-expression and was further decreased by inverse agonist AGN co-treatment compared to

control cells (Fig. 6E). Basal RARE reporter activity, however, was increased when endogenous *Zac1* or *RARγ* expression was inhibited by siRNA (Fig. 6G). Inhibition of *RARα* expression had no effect, attesting to the importance of *RARγ* (Fig. 6G); specificity of siRNAs is demonstrated in Figs. 6H, I.

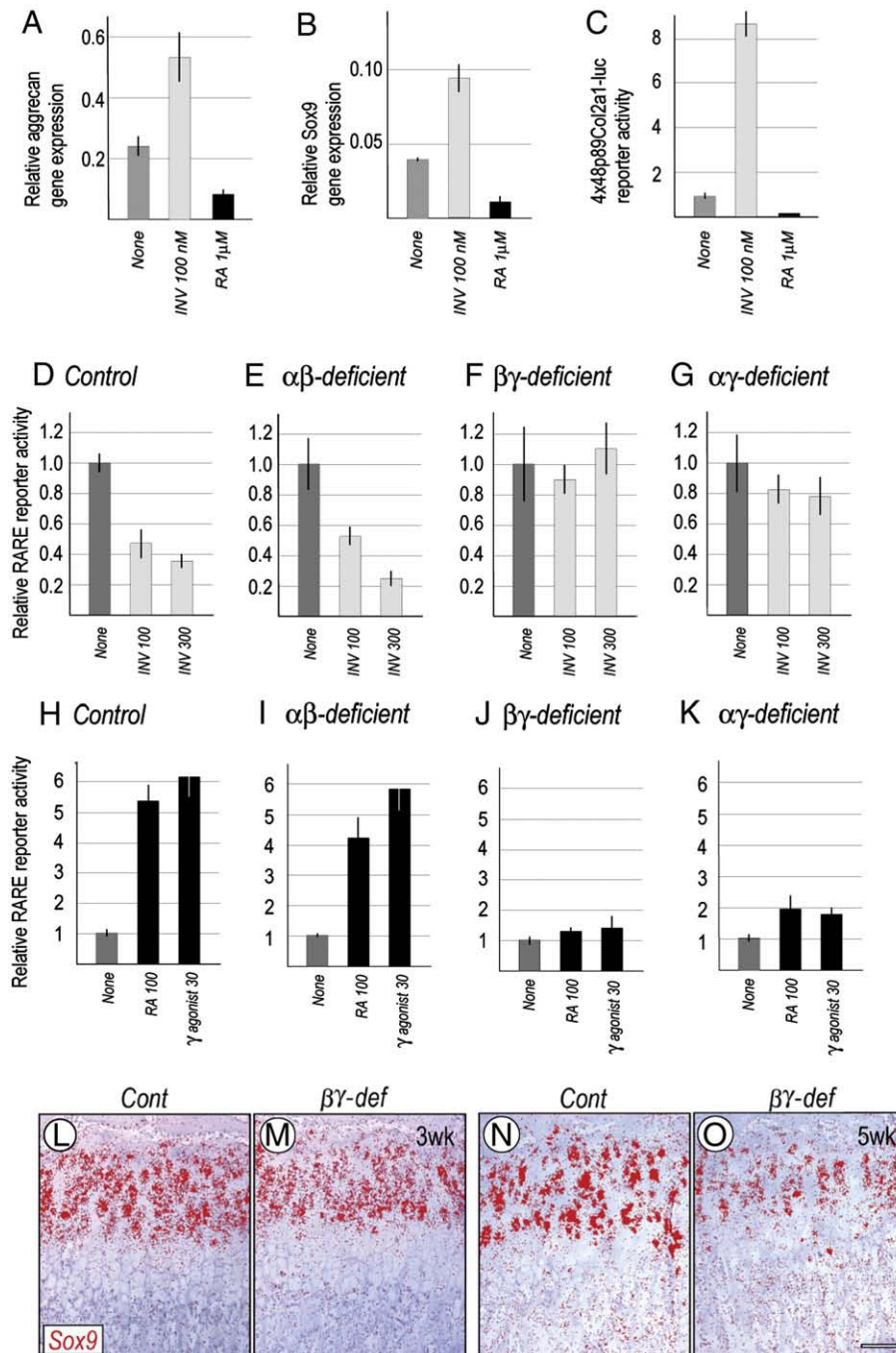
#### Regulation of proteoglycan expression

How could the apparent repressor function of RARs favor aggrecan gene expression? A reasonable possibility is that ligand-less RARs stimulate expression of *Sox 9* which is a critical regulator of



chondrogenesis (Bi et al., 1999) and is required for aggrecan expression (Bridgewater et al., 2003; Lefebvre and Smits, 2005). To assess this possibility, primary semi-confluent chondrocyte cultures were treated with 100 nM of inverse agonist (INV) for 2 days; for comparison, companion cultures were treated with a standard agonist (RA). Whole RNAs were processed for RT-PCR. Inverse agonist treatment did cause a concurrent stimulation of aggrecan and Sox9 expression (Figs. 7A, B), while RA inhibited both (Figs. 7A, B). The same trends were observed in cultures transfected with a Sox reporter construct that contains 4×48 bp Sox-binding *Col2a1*

enhancer (Zhou et al., 1998) (Fig. 7C). To verify that these pharmaceutical agents modulated RAR function at the genomic level and not via other pathways (Aggarwal et al., 2006; Alsayed et al., 2001), control and RAR-deficient chondrocyte cultures in standard medium were transfected with an RARE reporter plasmid and treated with inverse agonist, RA or RAR $\gamma$ -selective agonist. In control cultures, the inverse agonist markedly decreased baseline reporter activity (Fig. 7D) as seen above (see Fig. 6C), while RA or RAR $\gamma$ -selective agonist markedly stimulated it (Fig. 7H). The same results were obtained with RAR $\alpha$ /RAR $\beta$ -deficient cultures (Figs. 7E,



**Fig. 7.** Analyses of aggrecan and Sox9 expression levels and patterns. (A–C) Aggrecan and Sox9 expression levels (A, B) and 4×48 bp Sox reporter activity (C) in primary chondrocyte cultures treated with 100 nM inverse agonist (INV) or 1  $\mu$ M RA compared to untreated cultures (None). (D–K) RARE reporter activities in control, RAR $\alpha$ /RAR $\beta$ -, RAR $\beta$ /RAR $\gamma$ - and RAR $\alpha$ /RAR $\gamma$ -deficient chondrocyte cultures before (None) or after treatment with inverse agonist, RA or RAR $\gamma$ -selective agonist. All reporter assays were repeated 3–5 times and we obtained reproducible results. (L–O) In situ hybridization analysis of Sox9 expression in 3 week-old (L, M) and 5 week-old (N, O) proximal tibial growth plates from control (L and N) and RAR $\beta$ /RAR $\gamma$ -deficient (M and O) littermates.

l). However, no appreciable responses were seen in cultures deficient in *RAR $\beta$ /RAR $\gamma$*  (Figs. 7F, J) or *RAR $\alpha$ /RAR $\gamma$*  (Figs. 7G, K).

If the RARs regulate aggrecan expression via Sox9 as the above data imply, one would expect to see a decrease in Sox9 expression accompanying the decrease in aggrecan expression in *RAR $\beta$ /RAR $\gamma$* -deficient growth plates. In line with this prediction, there was a consistent and significant decrease in Sox9 transcripts in 3 and 5 week-old mutant versus control growth plates (Figs. 7L–O).

## Discussion

The study provides fundamentally new insights into RAR roles in skeletal growth. We find that the RARs are differentially expressed in different zones of prenatal and postnatal mouse long bone growth plates, with *RAR $\gamma$*  being the most abundantly expressed member and its expression prominently characterizing the upper zones. Concurrent deficiency in *RAR $\alpha$ /RAR $\gamma$*  or *RAR $\beta$ /RAR $\gamma$*  in cartilage causes concurrent defects in growth plates, most notably a reduction in their overall height and a decrease in chondrocyte proliferation and aggrecan expression and content. These growth plate defects are associated with disturbances in primary spongiosa and trabecular bone that are substandard compared to those seen in control littermates. Since chondrocyte proliferation and matrix deposition are amongst the most critical factors in dictating growth rates (Hunziker, 1994; Wilsman et al., 1996), it is very likely that deficiencies in those two parameters directly led to skeletal growth retardation in mutant mice. It is clear then that the RARs have significant, but previously unappreciated roles in growth plate chondrocyte function and exert local influence on skeletal growth. The data correlate quite well and greatly extend previous findings obtained with general dual *RAR $\alpha$ /RAR $\gamma$* - or *RAR $\beta$ /RAR $\gamma$* -null mice (Mendelsohn et al., 1994). The additional skeletal deformities seen in those mice and their neonatal lethality likely reflect broader systemic deficiencies due to general dual RAR deletion. On the other hand, the relatively mild defects in single RAR-null mutants reiterate the idea that other family members can compensate for lack of one RAR gene.

### Postnatal growth retardation

Our data show that the skeletal growth retardation affecting *RAR $\alpha$ /RAR $\gamma$* - and *RAR $\beta$ /RAR $\gamma$* -deficient mice became apparent around 3 weeks of age, continued into adulthood and was not compensated for throughout maturation. The apparent lack of growth retardation at a younger age correlates well with our observation that the mutant mice were born at expected Mendelian frequencies and may thus have been largely refractory to RAR deficiency during prenatal developmental stages. It has long been appreciated that prenatal skeletal growth is largely controlled by local autocrine and paracrine factors including Indian hedgehog, whereas systemic factors become important postnatally (Fowden, 1995; Kronenberg, 2003; Nilsson et al., 2005). For example, growth hormone has no apparent role in mouse embryonic skeletal growth despite the fact that its receptor is expressed, but GH-deficiency causes postnatal growth retardation starting around 2 weeks of age. Related studies showed that concurrent deficiency in GH and insulin-like growth factor 1 (or liver IGF-1 and acid labile subunit) cause severe mouse skeletal growth retardation starting around 3 weeks postnatally (Lupu et al., 2001; Yakar et al., 2002). A similar trend was observed in transgenic mice expressing the Achondroplasia mutant form of human FGF-R3; the mice were apparently normal during prenatal and neonatal stages, but became growth retarded starting around 4 weeks of age, with a concurrent decrease in growth plate chondrocyte proliferation (Naski et al., 1998). Clearly, skeletal growth is a complex process that obeys a multitude of common and distinct pathways and mechanisms during prenatal and postnatal life. Our data show that RAR deficiency is well tolerated during prenatal and neonatal growth, but cannot be

compensated and becomes deleterious to growth starting around 3 weeks of age by a combination of decreases in aggrecan production and deposition and chondrocyte proliferation. Whether the RAR deficiency affected the chondrocytes' ability to respond to, and be influenced by, systemic factors remains to be established.

### Retinoids and repressor function

Cartilage is unique among tissues for its avascular nature and growth plate cartilage is no exception, particularly in its upper zones. In fact, the growth plate is largely hypoxic and is quite sensitive to ablation of genes needed to cope with such an unusual metabolic status (Schipani et al., 2001). Given the absence of blood vessels, it is likely that the upper growth plate zones cannot receive liver-derived, blood-borne and protein-bound retinoid precursors needed for intracellular production of biologically-active retinoids (Blaner and Olson, 1994) and would thus be largely devoid of such retinoids. Indeed, von Schroeder and Heersche showed with a widely-used RARE reporter mouse line (Rossant et al., 1991) that the upper zones of mouse limb growth plates are virtually devoid of reporter activity and hence active retinoids, while reporter activity is detectable in the lower hypertrophic zone and other retinoid-rich organs (von Schroeder and Heersche, 1998). Our direct measurements of endogenous retinoic acid in upper growth plate zones by ultra-sensitive LC/MS/MS procedures (Kane et al., 2005, 2008) verify those observations (Fig. S4). Together, these studies suggest that RARs expressed in upper growth plate zones are largely devoid of active ligands and could primarily, if not exclusively, exert ligand-less repressor function. Growth plate defects seen in our *RAR $\alpha$ /RAR $\gamma$* - or *RAR $\beta$ /RAR $\gamma$* -deficient mice would thus result from absence of such repressor function.

Nuclear receptor-mediated transcriptional repression is as important as transcriptional activation in regulating developmental processes (Kurosawa et al., 1995; Mannervik et al., 1999). For example, *NCoR*- or *HDAC1*-null mice die during gestation (Jepsen et al., 2000; Lagger et al., 2002), and ablation of *HDAC4* (normally expressed by pre-hypertrophic chondrocytes) deranges growth plate chondrocyte maturation and causes premature hypertrophy (Vega et al., 2004). It is conceivable then that ligand-less RARs expressed in upper growth plate zones, and *RAR $\gamma$*  in particular, could cooperate with HDACs such as *HDAC4* to repress target genes, maintain chondrocyte function in those zones including aggrecan expression, and allow growth plate and skeletal growth to proceed at normal rates. Interestingly, our data indicate that Sox9 gene expression is lower in *RAR $\beta$ /RAR $\gamma$* -deficient growth plates and is stimulated by inverse agonist treatment in cultured chondrocytes (along with a stimulation in proteoglycan synthesis). A recent relevant report is showing that ligand-bound RARs or TNF $\alpha$  suppress cartilage-matrix gene expression by limiting availability of p300, an essential co-factor for Sox9 activity (Rockel et al., 2008). The findings suggest that p300 available for activation of Sox9 increases when RARs are ligand-less. Thus, a normal *in vivo* role of RAR repressor function in growth plate could be to favor Sox 9 expression and function together with p300 and promote the chondrocyte phenotype. A similar RAR repressor function, largely mediated by *RAR $\alpha$* , operates during limb mesenchymal chondrogenic cell differentiation and is required for initial up-regulation of Sox 9 gene expression (Weston et al., 2002). These authors suggested that *RAR $\alpha$* -dependent repressor function would directly block expression of "gene X", a negative regulator of Sox9 gene expression. Though the nature of gene X in chondrogenesis remains unknown, progress has been made in other systems. For instance, Jepsen et al. reported recently that *NCoR-2/SMRT* and ligand-less RARs cooperate to directly repress expression of histone demethylase *JMJD3* and in so doing, allow normal spatio-temporal progression of differentiation of neural stem cells into neurons (Jepsen et al., 2007).



### *Zac1 as a potential co-repressor*

While the nature of putative direct targets of RAR repression in growth plate cartilage also awaits further work, our current data have identified *Zac1* as a possible RAR co-factor. Our data not only show that the *Zac1* expression pattern is remarkably similar to that of *RARγ* in growth plate, but also that: *Zac1* functionally associates with *RARγ*; *Zac1* over-expression further boosts proteoglycan production in retinoid-free chondrocyte cultures; and *Zac1* and *RARγ* co-operate to reduce transcription rates as revealed by RARE reporter assays. As noted above, *Zac1* is an essential factor for many developmental processes (Spengler et al., 1997), and *Zac1*-null mice die at birth and exhibit significant defects including growth retardation and altered bone formation (Varrault et al., 2006). These authors performed a detailed *in silico* network gene analysis, carried out direct promoter and gene expression tests, and concluded that *Zac1* is upstream of a number of systemic genes, including *Igf2/H19*, *Cdkn1c* and *Dlk1*, that would together control embryonic growth. Since the growth plates of the *Zac1*-null mice were not studied, the authors did not clarify what consequences the general ablation of *Zac1* had for local chondrocyte and/or osteoblast function. Based on our data, we may surmise that absence of *Zac1* could have diminished RAR repressor function in the growth plate, should have affected the same phenotypic traits deranged by conditional RAR ablation such as aggrecan expression, and may even have had additional systemically-driven consequences due to substandard expression of upstream genes.

Our two hybrid tests suggest that *Zac1* and *RARγ* exhibit functional interaction, and this may possibly reflect physical interaction. Similar to the RARs (Chambon, 1996), *Zac1* is distinguished into several functional domains: an N-terminal zinc finger region; a central proline-rich region; a small and highly-conserved motif rich in hydrophobic amino acids (termed  $\Phi XX\Phi\Phi$  motif or NR box); and a C-terminal P/E-rich domain (Spengler et al., 1997). Using pull-down assays, Huang et al. showed that *Zac1* interacts with the AF2 domain of androgen, estrogen and thyroid hormone receptors (Huang and Stallcup, 2000), and it is possible that *Zac1* could interact with the same domain in RARs. Interestingly, our *in silico* analysis has not yet identified a consensus sequence in *Zac1* for interaction with HDACs (unpublished data). Thus, possible *Zac1*/RAR complexes may recruit HDACs indirectly by association with other HDAC-binding factors or may use alternative strategies, including SUMOylation as shown for NCoR1 (Tiefenbach et al., 2006).

### *Derangement of growth plate function*

The concurrent decreases in aggrecan expression, chondrocyte proliferation and trabecular bone deposition point to multiple important contributions of RARs in proper growth plate functioning. As our *in vivo* and *in vitro* data suggest, it is plausible that decreased RAR function may have led to decreased *Sox 9* gene expression which would in turn cause decreased aggrecan expression and substandard matrix deposition. A very interesting and far-reaching implication of these findings and conclusions is that experimental stimulation of RAR repressor function in cartilage by genetic means or pharmacological agents (such as the one used in our study) could actually have therapeutic value and could be used to stabilize or improve the phenotype and matrix production of chondrocytes including articular chondrocytes.

What could account for the other growth plate changes in RAR-deficient mice? Chondrocyte proliferation is obviously essential for skeletal development and growth and counterbalances the loss of hypertrophic chondrocytes occurring at the chondro-osseous border. Chondrocyte proliferation is controlled by systemic factors (Nilsson et al., 2005) and local mechanisms such as those regulated by Indian hedgehog (*Ihh*) (Shimo et al., 2004; St-Jacques et al., 1999). Interestingly, treatment of growth plate chondrocyte cultures with

retinoic acid (that induces RAR transcriptional activity) was found to strongly suppress *Ihh* expression (Wu et al., 2002). Thus, it is possible that under normal circumstances ligand-less RAR repressor function in upper growth plate zones may help to maintain physiologic *Ihh* expression levels.

Similarly, it remains to be clarified what caused the presence of substandard trabecular bone in the *RARα*/*RARγ*- or *RARβ*/*RARγ*-deficient mice. As pointed out above, it has long been appreciated that normal growth plate function and normal matrix composition are required for trabecular bone deposition. A first clue into such previously unsuspected paradigm came from the work of Poole and coworkers who originally showed that aggrecan persists in the calcified cartilage core of primary spongiosa trabeculae and is later removed by proteolytic action once the secondary spongiosa is laid down (Poole et al., 1982). A first genetic clue was provided by Jacenko and coworkers who showed that mice with altered growth plate collagen X function develop a skeleto-hematopoietic disease phenotype that includes a compressed growth plate, reduced primary spongiosa, and marrow hypoplasia (Gress and Jacenko, 2000). In an intriguing twist, it was realized recently that general deletion of *RARγ*, but not *RARα*, in mice actually causes bone marrow defects characterized by hematopoietic stem cell alterations and even a marked reduction in trabecular bone during aging (Purton et al., 2006; Walkley et al., 2007). It is pretty clear then that there are essential and intimate cross-talk interactions between growth plates and bone marrow required for physiologic progression of skeletal growth and development of hematopoietic and osteoprogenitor cells that can be deranged by malfunctioning of either growth plate or bone marrow.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.01.031.

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